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(71) Applicant: **Ajinomoto Co., Inc.**
Tokyo 104 (JP)

(72) Inventors:
- **Nakamura, Jun, c/o Ajinomoto Co., Inc.**
Kawasaki-shi, Kanagawa (JP)
- **Izui, Hiroshi, c/o Ajinomoto Co., Inc.**
Kawasaki-shi, Kanagawa (JP)
- **Moriguchi, Kayo, c/o Ajinomoto Co., Inc.**
Kawasaki-shi, Kanagawa (JP)
- **Kawashima, Hiroki, Ajinomoto Co., Inc.**
Kawasaki-shi, Kanagawa (JP)
- **Nakamatsu, Tsuyoshi,**
c/o Tokyo Denki University
Tokyo (JP)
- **Kurahashi, Osamu, c/o Ajinomoto Co., Inc.**
Kawasaki-shi, Kanagawa (JP)

(74) Representative: **HOFFMANN - EITLE**
Patent- und Rechtsanwälte
Arabellastrasse 4
81925 München (DE)

(54) Method for producing L-glutamine by fermentation and L-glutamine producing bacterium

(57) L-Glutamine is produced by culturing a coryneform bacterium which has L-glutamine producing ability and has been modified so that its intracellular glutamine synthetase activity should be enhanced, preferably which has been further modified so that its intracellular glutamate dehydrogenase activity should be enhanced, in a medium to produce and accumulate L-glutamine in the medium and collecting the L-glutamine.

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Description

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates to an L-glutamine producing bacterium belonging to coryneform bacteria and a method for producing L-glutamine. L-Glutamine is an industrially useful amino acid as an ingredient of seasonings, liver function promoting agents, amino acid transfusions, comprehensive amino acid preparations and so forth.

Related Art

[0002] In order to produce L-amino acids by fermentation, methods for improving microorganisms by breeding have been used abundantly. That is, since production ability of wild strains per se for L-amino acid production is extremely low in many cases, there have been known methods of imparting auxotrophy or analogue resistance by mutation or imparting mutation for metabolic regulation and methods utilizing a combination of these. Although L-glutamine can be obtained with an appropriate yield by the aforementioned methods, it is indispensable to further improve the fermentation yield in order to industrially produce L-glutamine at a low cost.

[0003] Further, the L-glutamine fermentation also suffers from the problem of by-production of L-glutamic acid. A method for solving this problem is proposed in, for example, Japanese Patent Laid-open Publication (Kokai) No. 3-232497. Although the production of L-glutamic acid can be suppressed to a certain extent by this method, there is still by-production of L-glutamic acid and the yield of L-glutamine is insufficient.

[0004] Since such improvements of L-glutamine producing bacteria as mentioned above utilize methods of treating a host bacterium with a mutagenizing agent or the like and selecting a strain showing improved productivity for L-glutamine from bacteria randomly incorporated with mutations, they require much labor and suffer from difficulties.

SUMMARY OF THE INVENTION

[0005] An object of the present invention is to find characteristics of coryneform bacteria providing improvement of L-glutamine productivity and suppression of by-production of L-glutamic acid, and thereby provide a method for producing L-glutamine utilizing a strain having such characteristics.

[0006] The inventors of the present invention assiduously studied in order to achieve the aforementioned object. As a result, they found that a strain of coryneform bacterium of which intracellular glutamine synthetase activity was enhanced showed more excellent L-glutamine producing ability and could markedly suppress the by-production of L-glutamic acid compared with strains showing the glutamine synthetase activity comparable to that of wild strains. Further, they found that production rate of L-glutamine was improved by simultaneously enhancing glutamine synthetase activity and glutamate dehydrogenase activity. Furthermore, they successfully isolated a novel gene coding for glutamine synthetase and a novel gene coding for glutamine synthetase adenylyl transferase, and thus accomplished the present invention.

[0007] That is, the present invention provides the followings.

(1) A coryneform bacterium which has L-glutamine producing ability and has been modified so that its intracellular glutamine synthetase activity should be enhanced.

(2) The bacterium according to (1), wherein the glutamine synthetase activity is enhanced by increasing expression amount of a glutamine synthetase gene.

(3) The bacterium according to (2), wherein the expression amount of the glutamine synthetase gene is increased by increasing copy number of a gene coding for glutamine synthetase or modifying an expression control sequence of the gene so that expression of the gene coding for the intracellular glutamine synthetase of the bacterium should be enhanced.

(4) The bacterium according to (1), wherein the glutamine synthetase activity is enhanced by deficiency in activity control of intracellular glutamine synthetase by adenylylation.

(5) The bacterium according to (4), wherein the activity control of intracellular glutamine synthetase by adenylylation is defected by one or more of harboring glutamine synthetase of which activity control by adenylylation is defected, decrease of glutaimine synthetase adenylyl transferase activities in the bacterial cell and decrease of PII protein activity in the bacterial cell.

(6) The bacterium according to any one of (1) to (5), wherein the bacterium has been further modified so that its intracellular glutamate dehydrogenase activity should be enhanced.

(7) The bacterium according to (6), wherein the glutamate dehydrogenase activity is enhanced by increasing ex-

pression amount of a glutamate dehydrogenase gene.

(8) The bacterium according to (7), wherein the expression amount of the glutamate dehydrogenase gene is increased by increasing copy number of the gene coding for glutamate dehydrogenase or modifying an expression control sequence of the gene so that expression of the gene coding for the intracellular glutamate dehydrogenase of the bacterium should be increased.

(9) A method for producing L-glutamine, which comprises culturing a bacterium according to any one of (1) to (8) in a medium to produce and accumulate L-glutamine in the medium and collecting the L-glutamine.

(10) A DNA coding for a protein defined in the following (A) or (B):

(A) a protein that has the amino acid sequence of SEQ ID NO: 2,

(B) a protein that has the amino acid sequence of SEQ ID NO: 2 including substitution, deletion, insertion, addition or inversion of one or several amino acid residues and has glutamine synthetase activity.

(11) The DNA according to (10), which is a DNA defined in the following (a) or (b):

(a) a DNA containing the nucleotide sequence of the nucleotide numbers 659-1996 in the nucleotide sequence of SEQ ID NO: 1,

(b) a DNA that is hybridizable with the nucleotide sequence of the nucleotide numbers 659-1996 in the nucleotide sequence of SEQ ID NO: 1 or a probe that can be prepared from the sequence under the stringent conditions and codes for a protein having glutamine synthetase activity.

(12) A DNA coding for a protein defined in the following (C) or (D):

(C) a protein that has the amino acid sequence of SEQ ID NO: 3,

(D) a protein that has the amino acid sequence of SEQ ID NO: 3 including substitution, deletion, insertion, addition or inversion of one or several amino acid residues and has glutamine synthetase adenylyl transferase activities.

(13) The DNA according to (12), which is a DNA defined in the following (c) or (d):

(c) a DNA containing the nucleotide sequence of nucleotide numbers 2006-5200 in the nucleotide sequence of SEQ ID NO: 1,

(d) a DNA that is hybridizable with the nucleotide sequence of the nucleotide numbers 2006-5200 in the nucleotide sequence of SEQ ID NO: 1 or a probe that can be prepared from the sequence under the stringent conditions and codes for a protein having glutamine synthetase adenylyl transferase activities.

[0008] According to the present invention, the by-production of L-glutamic acid can be suppressed and the production efficiency of L-glutamine can be improved in the production of L-glutamine by fermentation utilizing coryneform bacteria. Further, the DNA of the present invention can be used for breeding of L-glutamine producing coryneform bacteria.

PREFERRED EMBODIMENTS OF THE INVENTION

[0009] Hereafter, the present invention will be explained in detail.

(1) Coryneform bacteria of the present Invention

[0010] In the present invention, "coryneform bacteria" include those having hitherto been classified into the genus *Brevibacterium*, but united into the genus *Corynebacterium* at present (*Int. J. Syst. Bacteriol., 41,* 255 (1981)), and include bacteria belonging to the genus *Brevibacterium* closely relative to the genus *Corynebacterium*. Examples of such coryneform bacteria are mentioned below.

Corynebacterium acetoacidophilum
Corynebacterium acetoglutamicum
Corynebacterium alkanolyticum
Corynebacterium callunae
Corynebacterium glutamicum
Corynebacterium lilium
Corynebacterium melassecola
Corynebacterium thermoaminogenes

Corynebacterium herculis
Brevibacterium divaricatum
Brevibacterium flavum
Brevibacterium immariophilum
Brevibacterium lactofermentum
Brevibacterium roseum
Brevibacterium saccharolyticum
Brevibacterium thiogenitalis
Brevibacterium ammoniagenes
Brevibacterium album
Brevibacterium cerium
Microbacterium ammoniaphilum

[0011] Specifically, the following strains can be exemplified.
Corynebacterium acetoacidophilum ATCC 13870
Corynebacterium acetoglutamicum ATCC 15806
Corynebacterium alkanolyticum ATCC 21511
Corynebacterium callunae ATCC 15991
Corynebacterium glutamicum ATCC 13020, 13032, 13060
Corynebacterium lilium ATCC 15990
Corynebacterium melassecola ATCC 17965
Corynebacterium thermoaminogenes AJ12340 (FERM BP-1539)
Corynebacterium herculis ATCC 13868
Brevibacterium divaricatum ATCC 14020
Brevibacterium flavum ATCC 13826, ATCC 14067, AJ12418 (FERM BP-2205)
Brevibacterium immariophilum ATCC 14068
Brevibacterium lactofermentum ATCC 13869
Brevibacterium roseum ATCC 13825
Brevibacterium saccharolyticum ATCC 14066
Brevibacterium thiogenitalis ATCC 19240
Brevibacterium ammoniagenes ATCC 6871, ATCC 6872
Brevibacterium album ATCC 15111
Brevibacterium cerium ATCC 15112
Microbacterium ammoniaphilum ATCC 15354

[0012] To obtain these strains, one can be provided them from, for example, the American Type Culture Collection (10801 University Boulevard, Manassas, VA 20110-2209, United States of America). That is, each strain is assigned its registration number, and one can request provision of each strain by utilizing its registration number. The registration numbers corresponding to the strains are indicated on the catalog of the American Type Culture Collection. Further, the AJ12340 strain was deposited on October 27, 1987 at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (currently, the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary (Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, postal code: 305-8566)) as an international deposit under the provisions of the Budapest Treaty, and received an accession number of FERM BP-1539. The AJ12418 strain was deposited on January 5, 1989 at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry as an international deposit under the provisions of the Budapest Treaty and received an accession number of FERM BP-2205.

[0013] In the present invention, "L-glutamine producing ability" means an ability to accumulate L-glutamine in a medium, when the coryneform bacterium of the present invention is cultured in the medium. This L-glutamine producing ability may be possessed by the bacterium as a property of a wild strain of coryneform bacteria or may be imparted or enhanced by breeding.

[0014] For imparting or enhancing the L-glutamine producing ability by breeding, there can be used the method of isolation of 6-diazo-5-oxo-norleucine resistant strain (Japanese Patent Laid-open Publication No. 3-232497), the method of isolation of purine analogue resistant and/or methionine sulfoxide resistant strain (Japanese Patent Laid-open Publication No. 61-202694), the method of isolation of α-ketomalonic acid resistant strain (Japanese Patent Laid-open Publication No. 56-151495), the method of imparting resistance to a peptide containing glutamic acid (Japanese Patent Laid-open Publication No. 2-186994) and so forth. As specific examples of coryneform bacteria having L-glutamine producing ability, the following strains can be mentioned.
Brevibacterium flavum AJ11573 (FERM P-5492, refer to Japanese Patent Laid-open Publication No. 56-151495) *Brevibacterium flavum* AJ12210 (FERM P-8123, refer to Japanese Patent Laid-open Publication No. 61-202694) *Brevibac-*

terium flavum AJ12212 (FERM P-8123, refer to Japanese Patent Laid-open Publication No. 61-202694) *Brevibacterium flavum* AJ12418 (FERM-BP2205, refer to Japanese Patent Laid-open Publication No. 2-186994) *Brevibacterium flavum* DH18 (FERM P-11116, refer to Japanese Patent Laid-open Publication No. 3-232497) *Corynebacterium melassecola* DH344 (FERM P-11117, refer to Japanese Patent Laid-open Publication No. 3-232497) *Corynebacterium glutamicum* AJ11574 (FERM P-5493, refer to Japanese Patent Laid-open Publication No. No. 56-151495)

[0015] The term "modified so that intracellular glutamine synthetase (henceforth also referred to as "GS") activity should be enhanced" means that the GS activity per cell has become higher than that of a non-modified strain, for example, a wild-type coryneform bacterium. For example, there can be mentioned a case where number of GS molecules per cell increases, a case where GS specific activity per GS molecule increases and so forth. Further, as a wild-type coryneform bacterium that serves as an object for comparison, for example, the *Brevibacterium flavum* ATCC 14067 can be mentioned. As a result of enhancement of intracellular GS activity, there are obtained an effect that the amount of L-glutamine accumulation in a medium increases, an effect that the by-production of L-glutamic acid decreases and so forth.

[0016] Enhancement of GS activity in a coryneform bacterium cell can be attained by enhancement of expression of a gene coding for GS. Increase of the expression amount of the gene can be attained by increasing copy number of the gene coding for GS. For example, a recombinant DNA can be prepared by ligating a gene fragment coding for GS with a vector functioning in the bacterium, preferably a multi-copy type vector, and introduced into a host having L-glutamine producing ability to transform it. Alternatively, the aforementioned recombinant DNA can be introduced into a wild-type coryneform bacterium to obtain a transformant, and then the transformant can be imparted with L-glutamine producing ability.

[0017] As the GS gene, any of genes derived from coryneform bacteria and genes derived from other organisms such as bacteria belonging to the genus *Escherichia* can be used. Among these, genes derived from coryneform bacteria are preferred in view of ease of expression.

[0018] As the gene coding for GS of coryneform bacteria, *glnA* has already been elucidated (*FEMS Microbiology Letters*, 81-88, *154*, 1997). Therefore, a GS gene can be obtained by PCR (polymerase chain reaction; refer to White, T.J. *et al.*, *Trends Genet.*, *5*, 185 (1989)) utilizing primers prepared based on the nucleotide sequence of the gene, for example, the primers mentioned in Sequence Listing as SEQ ID NOS: 4 and 5, and chromosomal DNA of coryneform bacterium as a template. Genes coding for GS of other microorganisms can be obtained in a similar manner.

[0019] The chromosomal DNA can be prepared from a bacterium, which is a DNA donor, by the method of Saito and Miura (refer to H. Saito and K. Miura, *Biochem. Biophys. Acta, 72*, 619 (1963), Text for Bioengineering Experiments, Edited by the Society for Bioscience and Bioengineering, Japan, pp.97-98, Baifukan, 1992), for example.

[0020] Incidentally, an isozyme often exists for an enzyme involved in an amino acid biosynthesis system. The inventors of the present invention successfully isolated and cloned a gene coding for an isozyme of GS of coryneform bacteria by utilizing homology with respect to the nucleotide sequence of the aforementioned *glnA* gene. This gene is referred to as "*glnA2*". The process for obtaining it will be described later. *glnA2* as well as *glnA* can be used for enhancement of the GS activity of coryneform bacteria.

[0021] If the GS gene amplified by the PCR method is ligated to a vector DNA autonomously replicable in a cell of *Escherichia coli* and/or coryneform bacteria to prepare a recombinant DNA and this is introduced into *Escherichia coli*, subsequent procedures become easy. Examples of the vector autonomously replicable in a cell of *Escherichia coli* include pUC19, pUC18, pHSG299, pHSG399, pHSG398, RSF1010, pBR322, pACYC184, pMW219 and so forth.

[0022] A vector that functions in coryneform bacteria means, for example, a plasmid that can autonomously replicate in coryneform bacteria. Specific examples thereof include the followings.

pAM330 (refer to Japanese Patent Laid-open Publication No. 58-67699)

pHM1519 (refer to Japanese Patent Laid-open Publication No. 58-77895)

[0023] Moreover, if a DNA fragment having an ability to make a plasmid autonomously replicable in coryneform bacteria is taken out from these vectors and inserted into the aforementioned vectors for *Escherichia coli*, they can be used as a so-called shuttle vector autonomously replicable in both of *Escherichia coli* and coryneform bacteria.

[0024] Examples of such a shuttle vector include those mentioned below. There are also indicated microorganisms that harbor each vector, and accession numbers thereof at the international depositories are shown in the parentheses, respectively.

pAJ655	*Escherichia coli* AJ11882 (FERM BP-136) *Corynebacterium glutamicum* SR8201 (ATCC 39135)
pAJ1844	*Escherichia coli* AJ11883 (FERM BP-137) *Corynebacterium glutamicum* SR8202 (ATCC 39136)
pAJ611	*Escherichia coli* AJ11884 (FERM BP-138)
pAJ3148	*Corynebacterium glutamicum* SR8203 (ATCC 39137)
pAJ440	*Bacillus subtilis* AJ11901 (FERM BP-140)
pHC4	*Escherichia coli* AJ12617 (FERM BP-3532)

[0025] These vectors can be obtained from the deposited microorganisms as follows. That is, microbial cells collected in their exponential growth phase are lysed by using lysozyme and SDS, and centrifuged at 30000 x g. The supernatant obtained from the lysate is added with polyethylene glycol, fractionated and purified by cesium chloride-ethidium bromide equilibrium density gradient centrifugation.

[0026] In order to prepare a recombinant DNA by ligating a GS gene and a vector that can function in a cell of coryneform bacterium, a vector is digested with a restriction enzyme corresponding to the terminus of the gene containing the GS gene. Ligation is usually performed by using a ligase such as T4 DNA ligase.

[0027] To introduce the recombinant DNA prepared as described above into a microorganism, any known transformation methods that have hitherto been reported can be employed. For instance, employable are a method of treating recipient cells with calcium chloride so as to increase the permeability of DNA, which has been reported for *Escherichia coli* K-12 (Mandel, M. and Higa, A., *J. Mol. Bid., 53*, 159 (1970)), and a method of preparing competent cells from cells which are at the growth phase followed by introducing the DNA thereinto, which has been reported for *Bacillus subtilis* (Duncan, C.H., Wilson, G.A. and Young, F.E., *Gene, 1*, 153 (1977)). In addition to these, also employable is a method of making DNA-recipient cells into protoplasts or spheroplasts, which can easily take up recombinant DNA, followed by introducing the recombinant DNA into the cells, which is known to be applicable to *Bacillus subtilis*, actinomycetes and yeasts (Chang, S. and Choen, S.N., *Molec. Gen. Genet., 168*, 111 (1979); Bibb, M.J., Ward, J.M. and Hopwood, O.A., *Nature, 274,* 398 (1978); Hinnen, A., Hicks, J.B. and Fink, G.R., *Proc. Natl. Sci. USA*, 75, 1929 (1978)). The transformation of coryneform bacteria can also be performed by the electric pulse method (Sugimoto *et al.*, Japanese Patent Laid-open No. 2-207791).

[0028] Increase of copy number of GS gene can also be achieved by introducing multiple copies of the GS gene into chromosomal DNA of coryneform bacteria. In order to introduce multiple copies of the GS gene into chromosomal DNA of coryneform bacteria, homologous recombination is carried out by using a sequence whose multiple copies exist in the chromosomal DNA as targets. As sequences whose multiple copies exist in the chromosomal DNA, repetitive DNA, inverted repeats existing at the end of a transposable element can be used. Also, as disclosed in Japanese Patent Laid-open No. 2-109985, it is possible to incorporate the GS gene into transposon, and allow it to be transferred to introduce multiple copies of the gene into the chromosomal DNA.

[0029] Enhancement of the GS activity can also be attained by, besides being based on the aforementioned gene amplification, replacing an expression control sequence of the GS gene on chromosomal DNA or plasmid, such as a promoter, with a stronger one. For example, *lac* promoter, *trp* promoter, *trc* promoter and so forth are known as strong promoters. Moreover, it is also possible to introduce nucleotide substitution for several nucleotides into a promoter region for the GS gene so that it should be modified into a stronger one, as disclosed in International Patent Publication WO00/18935. By such substitution or modification of promoter, expression of the GS gene is enhanced and thus GS activity is enhanced. Such modification of expression control sequence may be combined with the increase of copy number of the GS gene.

[0030] The substitution of expression control sequence can be performed, for example, in the same manner as the gene substitution using a temperature sensitive plasmid described later. Examples of the temperature sensitive plasmid of coryneform bacteria include p48K, pSFKT2 (refer to Japanese Patent Laid-open Publication No. 2000-262288 for the both), pHSC4 (refer to France Patent Laid-open Publication No. 2667875, 1992 and Japanese Patent Laid-open Publication No. 5-7491) and so forth. These plasmids can at least autonomously replicate at a temperature of 25°C, but cannot autonomously replicate at a temperature of 37°C in coryneform bacteria. Although pSFKT2 was used for the substitution for the promoter sequence of the GDH gene in the example mentioned later, gene substitution can be performed in a similar manner by using pHSC4 instead of pSFKT2. *Esctierlchia coli* AJ12571 harboring pHSC4 was deposited on October 11, 1990 at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (currently, the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary (Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, postal code: 305-8566)), and received an accession number of FERM P-11763. Then, it was transferred to an international deposit under the provisions of the Budapest Treaty on August 26, 1991, and received an accession number of FERM BP-3524.

[0031] Enhancement of the GS activity can be attained also by deficiency in regulation by the adenylylation of intracellular GS, besides based on the increase of expression amount of the GS gene described above. GS changes into an inactive form by adenylylation of a tyrosine residue in the amino acid sequence (*Proc. Natl. Acad. Sci. USA*, 642-649, (*58*) 1967; *J. Biol. Chem.*, 3769-3771, (*243*) 1968). Therefore, by defect of this adenylylation of GS, the intracellular GS activity can be enhanced. The defect of adenylylation used herein means not only substantially complete deregulation by the adenylylation but also such reduction of the adenylylation that the intracellular GS activity should be enhanced.

[0032] The adenylylation of GS is generally performed by adenylyl transferase (*Proc. Natl. Acad. Sci. USA*, 1703-1710, (*58*) 1967). It has been suggested that, in coryneform bacteria, the 405th tyrosine residue of the *glnA* gene product, which is represented by the sequence of Genebank accession Y13221, is adenylylated (FEMS Microbiology

Letters, 303- 310, 1999 (173)). This inactivation by the adenylylation of GS can be defected by introducing a mutation into the *glnA* gene so that the tyrosine residue should be replaced with another amino acid residue.

[0033] Further, the inactivation of GS by the adenylylation can also be defected by reducing the activities of intracellular glutamine synthetase adenylyl transferase (ATase). Although adenylyl transferase of coryneform bacteria had been unknown, the inventors of the present invention successfully isolated a gene coding for adenylyl transferase of coryneform bacteria, *glnE*. The process therefor will be described later.

[0034] To reduce the intracellular ATase activity of coryneform bacteria, there can be used, for example, a method of treating the coryneform bacteria by ultraviolet irradiation or with a mutagenizing agent used for usual mutagenesis treatment such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or a nitrous acid and selecting a mutant strain in which the ATase activity is reduced. Coryneform bacteria having reduced ATase activity can also be obtained by gene disruption, besides the mutagenesis treatment. That is, a coryneform bacterium can be transformed with a DNA containing a *glnE* gene modified with deletion of partial sequence of the gene coding for ATase so as not to produce ATase functioning normally (deletion type *glnE* gene), so that recombination between the deletion type *glnE* gene and the *glnE* gene on the chromosome should occur to disrupt the *glnE* gene on the chromosome. Such gene disruption by gene substitution utilizing homologous recombination has already been established, and there are methods utilizing a linear DNA. a plasmid that contains a temperature sensitive replication origin and so forth.

[0035] A *glnE* gene on host chromosome can be replaced with the deletion type *glnE* gene, for example, as follows. That is, a recombinant DNA is prepared by inserting a temperature sensitive replication origin, a mutant *glnE* gene and a marker gene for resistance to a drug such as chloramphenicol, and a coryneform bacterium is transformed with the recombinant DNA. Further, the transformant is cultured at a temperature at which the temperature sensitive replication origin does not function, and then the transformant strain can be cultured in a medium containing the drug to obtain a transformant strain in which the recombinant DNA is incorporated into the chromosomal DNA.

[0036] In such a strain in which recombinant DNA is incorporated into chromosomal DNA as described above, the mutant *glnE* gene is recombined with the *glnE* gene originally present on the chromosome, and the two fusion genes of the chromosomal *glnE* gene and the deletion type *glnE* gene are inserted into the chromosome so that the other portions of the recombinant DNA (vector segment, temperature sensitive replication origin and drug resistance marker) should be present between the two fusion genes. Therefore, the transformant strain expresses normal ATase, because the normal *glnE* gene is dominant in this state.

[0037] Then, in order to leave only the deletion type *glnE* gene on the chromosomal DNA, one copy of the *glnE* gene is eliminated together with the vector segment (including the temperature sensitive replication origin and the drug resistance marker) from the chromosomal DNA by recombination of two of the *glnE* genes. In this case, the normal *glnE* gene is left on the chromosomal DNA, and the deletion type *glnE* gene is excised from the chromosomal DNA, or to the contrary, the deletion type *glnE* gene is left on the chromosomal DNA, and the normal *glnE* gene is excised from the chromosome DNA. In the both cases, the excised DNA may be retained in the cell as a plasmid when the cell is cultured at a temperature at which the temperature sensitive replication origin can function. Subsequently, if the cell is cultured at a temperature at which the temperature sensitive replication origin cannot function, the *glnE* gene on the plasmid is eliminated together with the plasmid from the cell. Then, a strain in which *glnE* gene is disrupted can be obtained by selecting a strain in which the deletion type *glnE* gene is left on the chromosome using PCR, Southern hybridization or the like.

[0038] Further, the inactivation of GS by the adenylylation can also be canceled by reducing the intracellular activity of PII protein. It is known that the PII protein is also involved in the adenylylation of GS by ATase. The PII protein is a signal transfer protein for controlling the GS activity, and it is known to be uridylylated by uridylyl transferase (UTase). The uridylylated PII protein promotes deadenylylation of GS by ATase, and the deuridylylated PII protein promotes the adenylylation of GS by ATase.

[0039] It is reported that GS is highly adenylylated in a UTase deficient strain (*J. Bacteriology*, 569-577, (*134*) 1978). This phenotype of excessive adenylylation is suppressed by mutation of the PII protein (*J. Bacteriology*, 816-822, *(164)* 1985). That is, the inactivation of GS by the adenylylation can also be defected by reduction of PII protein activity. The reduction of PII protein activity means reduction of the function for promoting the adenylylation by ATase. The *glnB* gene coding for the PII protein of coryneform bacteria has been already isolated, and it is suggested that the suppression of GS by the adenylylation of GS is defected by deletion of the gene (*FEMS Microbiology Letters*, 303-310, (*173*) 1999).

[0040] To reduce the PII protein activity of coryneform bacteria, there can be used, for example, a method of treating the coryneform bacteria by ultraviolet irradiation or with a mutagenizing agent used for usual mutagenesis treatment such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or a nitrous acid and selecting a mutant strain in which the activity of PII protein is reduced. Coryneform bacteria having reduced PII protein activity can also be obtained by gene disruption, besides the mutagenesis treatment. That is, a coryneform bacterium can be transformed with DNA containing a *glnB* gene modified with deletion of partial sequence of the gene coding for PII protein so as not to produce PII protein functioning normally (deletion type *glnB* gene), so that recombination between the deletion type *glnB* gene and the *glnB* gene on the chromosome should occur to disrupt the *glnB* gene on the chromosome. Such gene destruction by

utilizing homologous recombination has already been established, and there are methods utilizing a linear DNA, a plasmid that contains a temperature sensitive replication origin and so forth.

[0041] A *glnB* gene on host chromosome can be replaced with the deletion type *glnB* gene, for example, as follows. That is, a recombinant DNA is prepared by inserting a temperature sensitive replication origin, a mutant *glnB* gene and a marker gene for resistance to a drug such as chloramphenicol, and a coryneform bacterium is transformed with the recombinant DNA. Further, the resultant transformant strain is cultured at a temperature at which the temperature sensitive replication origin does not function, and then the transformant strain can be cultured in a medium containing the drug to obtain a transformant strain in which the recombinant DNA is incorporated into the chromosomal DNA.

[0042] In such a strain in which recombinant DNA is incorporated into chromosomal DNA as described above, the mutant *glnB* gene is recombined with the *glnB* gene originally present on the chromosome, and the two fusion genes of the chromosomal *glnB* gene and the deletion type *glnB* gene are inserted into the chromosome so that the other portions of the recombinant DNA (vector segment, temperature sensitive replication origin and drug resistance marker) should be present between the two fusion genes. Therefore, the transformant expresses normal PII protein, because the normal *glnB* gene is dominant in this state.

[0043] Then, in order to leave only the deletion type *glnB* gene on the chromosomal DNA, one copy of the *glnB* gene is eliminated together with the vector segment (including the temperature sensitive replication origin and the drug resistance marker) from the chromosomal DNA by recombination of two of the *glnB* genes. In this case, the normal *glnB* gene is left on the chromosomal DNA, and the deletion type *glnB* gene is excised from the chromosomal DNA, or to the contrary, the deletion type *glnB* gene is left on the chromosomal DNA, and the normal *glnB* gene is excised from the chromosome DNA. In the both cases, the excised DNA may be stably retained in the cell as a plasmid when the cell is cultured at a temperature at which the temperature sensitive replication origin can function. Subsequently, if the cell is cultured at a temperature at which the temperature sensitive replication origin does not function, the *glnB* gene on the plasmid is eliminated together with the plasmid from the cell. Then, a strain in which *glnB* gene is disrupted can be obtained by selecting a strain in which the deletion type *glnB* gene is left on the chromosome using PCR, Southern hybridization or the like.

[0044] Elimination of the adenylylation of GS can also be attained by a combination of two or three items of such mutation of GS that should eliminate the aforementioned adenylylation, reduction of the ATase activity and reduction of the PII protein activity.

[0045] Although enhancement of the GS activity can also be realized by elimination of the adenylylation of GS by ATase, it may also be attained by a combination of it with the aforementioned means for increasing copy number of the GS gene or means for modifying an expression control sequence.

[0046] In order to efficiently produce L-glutamine by using the coryneform bacterium of the present invention, it is preferable to use a strain that has enhanced glutamate dehydrogenase (henceforth also referred to as "GDH") activity concurrently with the enhanced GS activity.

[0047] The term "modified so that intracellular GDH activity should be enhanced" means that the GDH activity per cell has become higher than that of a non-modified strain, for example, a wild-type coryneform bacterium. For example, there can be mentioned a case where number of GDH molecules per cell increases, a case where GDH specific activity per GDH molecule increases and so forth. Further, as a wild-type coryneform bacterium that serves as an object for comparison, for example, the *Brevibacterium flavum* ATCC 14067 can be mentioned. As a result of enhancement of intracellular GDH activity, there are obtained an effect that culture time of a coryneform bacterium having L-glutamine producing ability is shortened.

[0048] Enhancement of the GDH activity in a coryneform bacterium cell can be attained by enhancement of expression of a gene coding for GDH. Enhancement of the expression amount of the gene can be attained by increasing copy number of the gene coding for GDH. For example, a recombinant DNA can be prepared by ligating a gene fragment coding for GDH with a vector functioning in the bacterium, preferably a multi-copy type vector, and introduced into a host having L-glutamine producing ability to transform it. Alternatively, the aforementioned recombinant DNA can be introduced into a wild-type coryneform bacterium to obtain a transformant strain, and then the obtained transformant strain can be imparted with L-glutamine producing ability.

[0049] As the gene coding for GDH, any of genes derived from coryneform bacteria and genes derived from other organisms such as bacteria belonging to the genus *Escherichia* can be used. Among these, genes derived from coryneform bacteria are preferred in view of ease of expression.

[0050] Nucleotide sequence of a gene coding for GDH (*gdh* gene) of coryneform bacteria has already been elucidated (*Molecular Microbiology, 6* (3), 317-326 (1992)). Therefore, a GDH gene can be obtained by PCR utilizing primers prepared based on the nucleotide sequence, for example, the primers mentioned in Sequence Listing as SEQ ID NOS: 12 and 13, and chromosomal DNA of coryneform bacterium as a template. Genes coding for GDH of microorganisms other than coryneform bacteria can also be obtained in a similar manner.

[0051] The *gdh* gene can be introduced into coryneform bacteria in a manner similar to that used for the aforementioned GS gene.

[0052] In the coryneform bacterium of the present invention, activities of enzymes other than GS and GDH catalyzing reactions of the L-glutamine biosynthesis may be enhanced. Examples of the enzymes catalyzing reactions of the L-glutamine biosynthesis include isocitrate dehydrogenase, aconitate hydratase, citrate synthase, pyruvate dehydrogenase, phosphoenolpyruvate carboxylase, pyruvate carboxylase, pyruvate kinase, phosphofructokinase and so forth.

[0053] Further, activities of enzymes that catalyze reactions branching off from the L-glutamine biosynthesis pathway and producing compounds other than L-glutamine may be reduced or eliminated. Examples of the enzymes catalyzing such reactions include isocitrate lyase, α-ketoglutarate dehydrogenase, glutamate synthase and so forth.

(2) Production of L-glutamine using microorganism of the present invention

[0054] By culturing a coryneform bacterium obtained as described above in a medium to produce and accumulate L-glutamine in the medium and correcting the L-glutamine from the medium, L-glutamine can be efficiently produced and the by-production of L-glutamic acid can be suppressed.

[0055] In order to produce L-glutamine by using the coryneform bacterium of the present invention, culture can be performed in a conventional manner using a usual medium containing a carbon source, nitrogen source and mineral salts as well as organic trace nutrients such as amino acids and vitamins, as required. Either a synthetic medium or a natural medium may be used. Any kinds of carbon source and nitrogen source may be used so long as they can be utilized by a strain to be cultured.

[0056] As the carbon source, there are used sugars such as glucose, glycerol, fructose, sucrose, maltose, mannose, galactose, starch hydrolysate and molasses, and organic acids such as acetic acid and citric acid, and alcohols such as ethanol can also be used each alone or in a combination with other carbon sources.

[0057] As the nitrogen source, there are used ammonia, ammonium salts such as ammonium sulfate, ammonium carbonate, ammonium chloride, ammonium phosphate and ammonium acetate, nitrate salts and so forth.

[0058] As the organic trace nutrients, amino acids, vitamins, fatty acids, nucleic acids, those containing those substances such as peptone, casamino acid, yeast extract and soybean protein decomposition product and so forth are used. When an auxotrophic mutant that requires an amino acid or the like for its growth is used, it is preferable to supplement the required nutrient.

[0059] As the mineral salts, phosphates, magnesium salts, calcium salts, iron salts, manganese salts and so forth are used.

[0060] The culture is performed as aeration culture, while the fermentation temperature is controlled to be 20-45°C, and pH to be 5-9. When pH falls during the culture, the medium is neutralized by addition of calcium carbonate or with an alkali such as ammonia gas. A substantial amount of L-glutamine is accumulated in the culture broth after 10 hours to 120 hours of culture in such a manner as described above.

[0061] Collection of L-glutamine from the culture broth after the culture may be performed in a conventional manner. For example, after the cells were removed from the culture broth, L-glutamine can be collected by concentrating the broth to crystallize L-glutamine.

(3) DNA coding for protein having glutamine synthetase activity (*glnA2* gene) and DNA coding for protein having glutamine synthetase and adenylyl transferase activities (*glnE* gene) according to the present invention

[0062] The first DNA of the present invention is a gene coding for GS. The second DNA of the present invention is a gene coding for ATase. These genes can be obtained from a chromosome DNA library of *Brerribacterium lactofermentum* by hybridization using a partial fragment of a known *glnA* gene as a probe. The partial fragment of a known *glnA* gene can be obtained by PCR amplification using chromosome DNA of *Brevibacterium lactofermentum*, for example, *Brevibacterium lactofermentum* ATCC 13869 strain, as a template and the primers shown as SEQ ID NOS: 18 and 19.

[0063] Methods of production of genomic DNA library, hybridization, PCR, preparation of plasmid DNA, digestion and ligation of DNA, transformation and so forth for obtaining the DNA of the present invention and enhancement of the GS activity and GDH activity are described in Sambrook, J., Fritsh, E.F. and Maniatis, T., "Molecular Cloning", Cold Spring Harbor Laboratory Press, 1.21, 1989.

[0064] The nucleotide sequences of the aforementioned primers were designed based on the nucleotide sequence of a *glnA* gene of *Corynebacterium glutamicum* (GenBank accession Y13221). By using these primers, a DNA fragment containing a region corresponding to the nucleotide numbers 1921-2282 of the *glnA* gene (GenBank accession Y13221) can be obtained.

[0065] Examples of nucleotide sequence of DNA fragment containing *glnA2* according to the present invention, which is obtained as described above, and amino acid sequence that can be encoded by the sequence are shown as SEQ ID NO: 1. Further, only an amino acid sequence of protein having glutamine synthetase activity, which is encoded by *glnA2*, is shown in SEQ ID NO: 2.

[0066] Further, in the aforementioned DNA fragment, another ORF was found immediately downstream from ORF of the *glnA2* gene. Based on homology comparison with respect to known sequences, that ORF was expected to be a gene (*glnE*) coding for a protein having glutamine synthetase adenylyl transferase activities (ATase). Only the amino acid sequence of the protein having the ATase activity is shown as SEQ ID NO: 3.

[0067] Nucleotide sequences of the DNA fragments containing *glnA2* or *glnE* according to the present invention were clarified by the present invention. Therefore, they can be isolated from chromosomal DNA of *Brevibacterium lactofermentum* by the PCR method using primers produced based on the nucleotide sequences.

[0068] The first DNA of the present invention may be one coding for glutamine synthetase including substitution, deletion. insertion, addition or inversion of one or several amino acids at one or more sites, so long as the glutamine synthetase activity of the encoded protein is not defected. Although the number of "several" amino acids referred to herein differs depending on position or type of amino acid residues in the three-dimensional structure of the protein, it may be specifically 2 to 90, preferably 2 to 50, more preferably 2 to 20.

[0069] The second DNA of the present invention may be one coding for glutamine synthetase adenylyl transferase including substitution, deletion, insertion, addition or inversion of one or several amino acids at one or more sites, so long as the glutamine synthetase adenylyl transferase activities of the encoded protein are not defected. Although the number of "several" amino acids referred to herein differs depending on position or type of amino acid residues in the three-dimensional structure of the protein, it may be specifically 2 to 350, preferably 2 to 50, more preferably 2 to 20. Even in a case that the glutamine synthetase and adenylyl transferase activities are impaired, such a DNA fall within the scope of the present invention so long as it causes homologous recombination.

[0070] A DNA coding for the substantially same protein as the aforementioned GS or ATase can be obtained by, for example, modifying the nucleotide sequence of *glnA2* or *glnE* by means of the site-directed mutagenesis method so that one or more amino acid residues at a specified site should involve substitution, deletion, insertion, addition or inversion. A DNA modified as described above may also be obtained by a conventionally known mutagenesis treatment. The mutagenesis treatment includes a method of treating a DNA before the mutagenesis treatment in vitro with hydroxylamine or the like, and a method for treating a microorganism such as an genus *Escherichia* harboring a DNA before the mutagenesis treatment by ultraviolet irradiation or with a mutagenizing agent used for a usual mutagenesis treatment such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and nitrous acid.

[0071] A DNA coding for substantially the same protein as glutamine synthetase or glutamine synthetase adenylyl transferase can be obtained by expressing a DNA having such a mutation as described above in an appropriate cell, and investigating activity of an expressed product. A DNA coding for substantially the same protein as GS or ATase can also be obtained by isolating a DNA that is hybridizable with a probe having a nucleotide sequence comprising, for example, the nucleotide sequence corresponding to nucleotide numbers of 659 to 1996 or 2066 to 5200 of the nucleotide sequence shown in Sequence Listing as SEQ ID NO: 1, under the stringent conditions, and codes for a protein having the glutamine synthetase or a protein having the glutamine synthetase adenylyl transferase activity, from DNA coding for glutamine synthetase or glutamine synthetase and adenylyl transferase having a mutation or from a cell harboring it. The "stringent conditions" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent conditions are exemplified by a condition under which DNAs having high homology, for example, DNAs having homology of not less than 50% are hybridized with each other, but DNAs having homology lower than the above are not hybridized with each other. Alternatively, the stringent conditions are exemplified by a condition under which DNAs are hybridized with each other at a salt concentration corresponding to an ordinary condition of washing in Southern hybridization, i.e., 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS, at 60°C.

[0072] As a probe, a partial sequence of the nucleotide sequence of SEQ ID NO: 1 can also be used. Such a probe may be prepared by PCR using oligonucleotides produced based on the nucleotide sequence of SEQ ID NO: 1 as primers, and a DNA fragment containing the nucleotide sequence of SEQ ID NO: 1 as a template. When a DNA fragment in a length of about 300 bp is used as the probe, the conditions of washing for the hybridization consist of, for example, 50°C, 2 x SSC and 0.1% SDS.

[0073] Genes that are hybridizable under such conditions as described above includes those having a stop codon in the genes, and those having no activity due to mutation of active center. However, such mutation can be easily removed by ligating each gene with a commercially available activity expression vector, and measuring the glutamine synthetase or glutamine synthetase adenylyl transferase activities. The glutamine synthetase activity can be measured by, for example, the method described in *Methods in Enzymology*, Vol. XVIIA, 910-915, ACADEMIC PRESS (1970), and the glutamine synthetase adenylyl transferase activities can be measured by, for example, the method described in *Methods in Bnzymology,* Vol. XVIIA, 922-923, ACADEMIC PRESS (1970). Even a DNA coding for glutamine synthetase adenylyl transferase of which activities are reduced or deleted can also be used in the present invention.

[0074] Specific examples of the DNA coding for a protein substantially the same as GS include DNA coding for a protein that has homology of preferably 80% or more, more preferably 85% or more, still more preferably 90% or more, with respect to the amino acid sequence shown as SEQ ID NO: 2 and has GS activity. Specific examples of the DNA

coding for a protein substantially the same as ATase include DNA coding for a protein that has homology of preferably 65% or more, more preferably 80% or more, still more preferably 90% or more, with respect to the amino acid sequence shown as SEQ ID NO: 3 and has ATase activity.

Best Mode for Carrying out the Invention

[0075] Hereafter, the present invention will be explained more specifically with reference to the following examples.

Example 1: Evaluation of GS gene-amplified strain

(1) Cloning of *glnA* gene of coryneform bacterium

[0076] The *glnA* sequence of *Corynebacterium glutamicum* had been already clarified (*FEMS Microbiology Letters,* 81-88, (*154*) 1997). Based on the reported nucleotide sequence, the primers shown in Sequence Listing as SEQ ID NOS: 4 and 5 were synthesized, and a *glnA* fragment was amplified by the PCR method using chromosome DNA of *Brevibacterium flavum* ATCC 14067 strain as a template.

[0077] The chromosomal DNA of *Brevibacterium flavum* ATCC 14067 strain was prepared by using Bacterial Genome DNA Purification Kit (Advanced Genetic Technologies Corp.). PCR was performed for 30 cycles each consisting of reactions at 94°C for 30 seconds for denaturation, at 55°C for 15 seconds for annealing and 72°C for 2 minutes for extension by using Pyrobest DNA Polymerase (Takara Shuzo).

[0078] The produced PCR product was purified in a conventional manner, digested with a restriction enzyme *Sal*I, ligated with pMW219 (Nippon Gene) digested with *Sal*I by using a ligation kit (Takara Shuzo), and used to transform competent cells of *Escherichia coli* JM109 (Takara Shuzo). The cells were plated on L medium containing 10 μg/ml of IPTG, 40 μg/ml of X-Gal and 25 μg/ml of kanamycin and cultured overnight. Then, the appeared white colonies were picked up and separated into single colonies to obtain transformants.

[0079] Plasmids are prepared from the transformants by the alkali method, and a plasmid in which the *glnA* gene was inserted into the vector was designated as pMW219GS.

(2) Construction of plasmid having *glnA* and replication origin of coryneform bacteria

[0080] Further, in order to construct a plasmid having the *glnA* gene and a replication origin of coryneform bacteria, the plasmid pHK4 (refer to Japanese Patent Laid-open Publication No. 5-7491) containing replication origin of the plasmid pHM1519 (*Agric. Biol. Chem., 48,* 2901-2903 (1984)) that had been already obtained and was autonomously replicable in coryneform bacteria was digested with restriction enzymes *Bam*HI and *Kpn*I to obtain a gene fragment containing the replication origin. The obtained fragment was blunt-ended by using DNA Blunt-ending Kit (Takara Shuzo) and inserted into the *Kpn*I site of pMW219GS using a *Kpn*I linker (Takara Shuzo). This plasmid was designated as pGS.

(3) Introduction of pGS into coryneform bacterium and evaluation of culture

[0081] An L-glutamine producing bacterium, *Brevibacterium flavum* AJ12418 (FERM BP-2205: refer to Japanese Patent Laid-open Publication No. 2-186994), was transformed with the plasmid pGS by the electric pulse method (refer to Japanese Patent Laid-open Publication No. 2-207791) to obtain a transformant. By using the obtained transformant AJ12418/pGS, culture for L-glutamine production was performed as follows.

[0082] Cells of AJ12418/pGS strain obtained by culture on a CM2B plate medium containing 25 μg/ml of kanamycin were inoculated into a medium containing 100 g of glucose, 60 g of $(NH_4)_2SO_4$, 2.5 g of KH_2PO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 350 μg of VB_1-HCl, 4 μg of biotin, 200 mg of soybean hydrolysates and 50 g of $CaCO_3$ in 1 L of pure water (adjusted to pH 6.8 with NaOH) and cultured at 31.5°C with shaking until the sugar in the medium was consumed.

[0083] After the completion of the culture, the amount of accumulated L-glutamine in the culture broth was analyzed by liquid chromatography for appropriately diluted culture broth. CAPCELL PAK C18 (Shiseido) was used as a column, and the sample was eluted with an eluent containing 0.095% phosphoric acid, 3.3 mM heptanesulfonic acid and 5% acetonitrile in 1 L of distilled water. The accumulated L-glutamine amount was analyzed based on variation of absorbance at 210 nm. The results of this analysis are shown in Table 1.

Table 1

Strain	L-Gln (g/L)	L-Glu (g/L)	culture time (hr)
AJ12418	38.4	0.7	70

Table 1 (continued)

Strain	L-Gln (g/L)	L-Glu (g/L)	culture time (hr)
AJ12418/pGS	45.1	0.02	82

[0084] In the pGS-introduced strain, accumulation of L-glutamine (L-Gln) was markedly improved, and by-production of L-glutamic acid (L-Glu) was considerably suppressed. From these results, it was demonstrated that enhancement of GS was effective for improvement of yield in the production of L-glutamine. The data for the enzymatic activity of GS are shown in Table 2 of Example 2.

Example 2: Evaluation of GS adenylylation site-modified strain

(1) Construction of adenylylation site-modified plasmid

[0085] The adenylylation site of *glnA* gene product of coryneform bacteria had been already clarified (*FEMS Microbiology Letters*, 303-310, (*173*) 1999). Therefore, an adenylylation site-modified strain was obtained by replacing the *glnA* gene on the chromosome with a *glnA* gene of which adenylylation site was modified. Specific procedures will be described below.

[0086] First, PCR was performed by using chromosome DNA of *Brevibacterium flavum* ATCC 14067 strain as a template and the synthetic DNAs shown in Sequence Listing as SEQ ID NOS: 6 and 7 as primers to obtain an amplification product for the N-terminus side of the *glnA* gene. Separately, in order to obtain an amplification product for the C-terminus side of the *glnA* gene, PCR was performed by using chromosome DNA of *Brevibacterium flavum* ATCC 14067 strain as a template and the synthetic DNAs shown in Sequence Listing as SEQ ID NOS: 8 and 9 as primers. Since mismatches were introduced into the sequences shown in Sequence Listing as SEQ ID NOS: 7 and 8, a mutation was introduced into the terminal portion of each of the amplification products. Then, in order to obtain a *glnA* gene fragment introduced with a mutation, PCR was performed by using the aforementioned gene products for N- and C-terminus sides of *glnA* mixed in equimolar amounts as a template and the synthetic DNAs shown in Sequence Listing as SEQ ID NOS: 10 and 11 as primers to obtain a *glnA* gene amplification product introduced with a mutation at the adenylylation site. The produced PCR product was purified in a conventional manner, digested with *Hind*I and inserted into the *Hind*II site of pHSG299 (Takara Shuzo). This plasmid was designated as pGSA.

(2) Construction of adenylylation site-modified strain and evaluation of culture

[0087] Since the above pGSA does not contain a region that enables its autonomous replication within cells of coryneform bacteria, when a coryneform bacterium is transformed with this plasmid, a strain in which the plasmid is incorporated into chromosome by homologous recombination is obtained as a transformant although it occurs at an extremely low frequency.

[0088] The L-glutamine producing bacterium, *Brevibacterium flavum* AJ12418, was transformed with the plasmid pGSA at a high concentration by the electric pulse method (refer to Japanese Patent Laid-open Publication No. 2-207791), and transformants were obtained by using kanamycin resistance as a marker. Then, these transformants were subcultured and strains that became kanamycin sensitive were obtained. Further, the sequences of *glnA* gene of the kanamycin sensitive strains were determined, and a strain in which the adenylylation site in the sequence was replaced with that region of *glnA* derived from pGSA was designated as QA-1. Culture for L-glutamine production was performed in the same manner as described in Example 1, (3) using AJ12418, AJ12418/pGS and QA-1 strains. The results are shown in Table 2.

Table 2

Strain	L-Gln (g/L)	GS activity (U/mg)	Culture time (hr)
AJ12418	39.0	0.030	70
AJ12418/pGS	46.1	0.067	81
QA-1	44.3	0.040	72

[0089] For the QA-1 strain, improvement of L-glutamine accumulation was observed compared with AJ12418.

[0090] The results for measurement of GS activity of these strains are also shown in Table 2. The GS activity was measured by adding a crude enzyme solution to a solution containing 100 mM imidazole-HCl (pH 7.0), 0.1 mM NH_4Cl, 1 mM $MnCl_2$, 1 mM phosphoenolpyruvic acid, 0.3 mM NADH, 10 U of lactate dehydrogenase, 25 U of pyruvate kinase,

1 mM ATP and 10 mM MSG and measuring variation of absorbance at 340 nm at 30°C referring to the method described in *Journal of Fermentation and Bioengineering*, Vol. *70*, No. 3, 182-184, 1990. For the measurement of blank, the aforementioned reaction solution not containing MSG was used. The crude enzyme solution was prepared by separating cells from the aforementioned culture broth by centrifugation, washing the cells with 100 mM imidazole-HCl (pH 7.0), sonicating the cells and removing undisrupted cells and unsoluble protein by centrifugation. Protein concentration of the crude enzyme solution was quantified with Protein Assay (Bio-Rad) by using bovine serum albumin as a standard sample.

Example 3: Evaluation of GDH gene-amplified strain

(1) Construction of *gdh*-amplified strain and evaluation of culture

[0091] Construction of a plasmid pGDH into which the *gdh* gene of coryneform bacteria was cloned was performed as follows. First, chromosome DNA of *Brevibacterium lactofermentum* ATCC 13869 strain was extracted, and PCR was performed by using the chromosome DNA as a template and the synthetic DNAs shown in Sequence Listing as SEQ ID NOS: 12 and 13 as primers. The obtained DNA fragment was blunt-ended and inserted into the SmaI site of pHSG399 (Takara Shuzo). This plasmid was designated as pHSG399GDH.

[0092] Then, a replication origin derived from the plasmid pHM1519 (*Agric. Biol. Chem.*, *48*, 2901-2903 (1984)) that could autonomously replicate in coryneform bacteria was introduced into the *Sal*I site of pHSG399GDH. Specifically, the aforementioned pHK4 was digested with restriction enzymes BamHI and *Kpn*I to obtain a gene fragment containing the replication origin, and the obtained fragment was blunt-ended and inserted into the *Sal*I site of pHSG399GDH by using an *Sal*I linker (Takara Shuzo). This plasmid was designated as pGDH.

[0093] The L-glutamine producing bacterium, *Brevibacterium flavum* AJ12418 strain, was transformed with pGDH to obtain a transformant. Culture for L-glutamine production was performed by the method described in Example 1 using the obtained transformant AJ12418/pGDH. The results are shown in Table 3. In the GDH-enhanced strain, yield of L-glutamine decreased and by-production of L-glutamic acid increased, but culture time was considerably shortened.

Table 3

Strain Strain	L-Gln (g/L) L-Gln (g/L)	L-Glu (g/L) L-Glu (g/L)	Culture time (hr)
AJ12418	38.8	0.7	70
AJ12418/pGDH	29.5	12.0	55

Example 4: Construction and evaluation of strain in which GS and GDH are enhanced simultaneously

(1) Construction of *gdh* promoter-modified plasmid

[0094] Chromosomal DNA of *Brevibacterium flavum* ATCC 14067 strain was extracted, and PCR was performed by using the chromosomal DNA as a template and the synthetic DNAs shown in Sequence Listing as SEQ ID NOS: 14 and 15 as primers. The obtained DNA fragment was digested with restriction enzymes *Stu*I and *Pvu*II and inserted into the *Sma*I site of pHSG399. This plasmid was digested with a restriction enzyme *Sac*I to obtain a DNA fragment containing the *gdh* promoter and a partial fragment of the *gdh* gene, and it was inserted into the *Sac*I site of pKF19k (Takara Shuzo). This plasmid was designated as pKF19GDH.

[0095] A mutation was introduced into the promoter region by using Mutan-Super Express Km (Takara Shuzo). LA-PCR was performed by using pkF19GDH as a template, a selection primer attached to Mutan-super Express Km and a 5'-end phosphorylated synthetic DNA shown in Sequence Listing as SEQ ID NO: 16 or 17 as a primer for mutagenesis. The reaction product was purified by ethanol precipitation, and competent cells of *Escherichia coli* MV1184 (Takara Shuzo) were transformed with the product to obtain transformants.

[0096] Plasmids were extracted from the transformants, and sequences of the *gdh* promoter region were determined. Among these, those having the sequences shown in Table 4 were designated as pKF19GDH1 and pKF19GDH4. It is expected that the GDH activity can be improved by about 3 times by replacing the *gdh* promoter sequence with that of pKF19GDH1 type, or by about 5 times by replacing the *gdh* promoter sequence with that of pKF19GDH4 type, compared with *gdh* having a promoter of a wild-type (refer to International Patent Publication WO00/18935).

[0097] These plasmids were digested with a restriction enzyme *Sac*I to obtain a DNA fragment containing the *gdh* promoter and a partial fragment of the *gdh* gene, and it was inserted into the *Sac*I site of pSFKT2 (refer to Japanese Patent Laid-open Publication No. 2000-262288). These plasmids were designated as pSFKTGDH1 and pSFKTGDH4, respectively. pSFKT2 was a derivative of the plasmid pAM330 derived from the *Brevibacterium lactofermentum* ATCC 13869 strain, and it is a plasmid of which autonomous replication in coryneform bacteria has become temperature

sensitive.

Table 4

Plasmid	*gdh* promoter sequence
pKF19GDH	TGGTCAtatctgtgcgacgctgcCATAAT (SEQ ID NO: 20)
pKF19GDH1	TGGTCAtatctgtgcgacgctgcTATAAT (SEQ ID NO: 21)
pKF19GDH4	TTGCCAtatctgtgcgacgctgcTATAAT (SEQ ID NO: 22)

(2) Introduction of *gdh* promoter mutation into chromosome

[0098] A mutation was introduced into the *gdh* promoter sequence on chromosome as follows. First, the QA-1 strain was transformed with the plasmid pSFKTGDH1 or pSFKTGDH4 by the electric pulse method to obtain a transformant, respectively. After the transformation, culture was performed at 25°C. Then, these transformants were cultured at 34°C, and strains showing kanamycin resistance at 34°C were selected. Since the aforementioned plasmids cannot autonomously replicate at 34°C, only those in which these plasmids were integrated into chromosome by homologous recombination show kanamycin resistance. Further, the strains in which these plasmids were integrated into chromosome were cultured in the absence of kanamycin, and strains that became kanamycin sensitive were selected. Among those, strains in which the same mutation as that of pSFKTGDH1 or pSFKTGDH4 was introduced into the *gdh* promoter region on the chromosome were designated as QB-1 and QB-4, respectively.

(3) Construction of *gdh* gene-amplified strain and measurement of GDH activity

[0099] The L-glutamine producing bacterium, *Brevibacterium flavum* QA-1 strain, was transformed with the plasmid pGDH described in Example 3, (2) to obtain a transformant. Culture for L-glutamine production was performed by the method described in Example 1 using the obtained transformant QA-1/pGDH. The GDH activity was measured by adding a crude enzyme solution to a solution containing 100 mM Tris-HCl (pH 7.5), 20 mM NH_4Cl, 10 mM α-ketoglutaric acid and 0.25 mM NADPH and measuring change of absorbance at 340 nm referring to *Mol. Microbiology,* 317-326 (*6*) 1992. The crude enzyme solution was prepared by separating cells from the aforementioned culture broth by centrifugation, washing the cells with 100 mM Tris-HCl (pH 7.5), sonicating the cells and removing undisrupted cells by centrifugation. Protein concentration of the crude enzyme solution was quantified with Protein Assay (Bio-Rad) by using bovine serum albumin as a standard sample. The results are shown in Table 5.

[0100] As for yield of L-glutamine, the GDH promoter-modified strains, QB-1 and QB-4, showed high yield. Further, the QA-1/pGDH strain also showed higher yield than that obtained with the AJ12418 strain. The culture time of the QA-1/pGDH strain was the shortest. The by-production of L-glutamic acid was markedly improved in the QB-1 and QB-4 strains. From these results, it was demonstrated that the simultaneous enhancement of GS and GDH was effective for improvement of yield of L-glutamine and shortening of culture time.

Table 5

Strain Strain	L-Gln (g/L)	L-Glu (g/L)	Culture time (hr)	GDH activity (U/mg)
AJ12418	40.5	0.8	68	1.6
QA-1/PGDH	47.9	1.0	60	15.2
QB-1	50.5	0.1	65	4.1
QB-4	50.0	0.3	65	9.6

Example 5: Acquisition of gene coding for isozyme of GS

[0101] In the paper that reported acquisition of *glnA* of *Corynebacterium glutamicum* (*FEMS Microbiol. Letter, 154* (1997) 81-88), it is described that a ΔglnA-disrupted strain became to show glutamine auxotrophy and lost the GS activity, and it also reported data showing results of Southern blotting and suggesting existence of an isozyme. Further, "Amino Acid Fermentation", Japan Science Societies Publication (Gakkai Shuppan Center), pp.232-235 describes that there are two kinds of GS for *Corynebacterium glutamicum*. Therefore, it was attempted to obtain a gene coding for the second GS isozyme.

(1) Preparation of probe

[0102] A gene coding for an isozyme of GS (*glnA2*) was obtained by colony hybridization. First, PCR was performed by using the primers shown in Sequence Listing as SEQ ID NOS: 18 and 19 and chromosomal DNA of the *Brevibacterium lactofermentum* ATCC 13869 strain as a template to obtain a partial fragment of the *glnA* gene. This DNA fragment was labeled by using DIG-High Prime DNA Labeling & Detection Starter Kit I (Boehringer Mannheim) and used as a probe.

(2) Colony hybridization

[0103] Chromosomal DNA of the *Brevibacterium lactofermentum* ATCC 13869 strain was extracted and partially digested with a restriction enzyme *Sau3AI*, and the obtained DNA fragment was inserted into the *BamHI* site of the vector of pHSG299 and used to transform the *Escherichia coli* JM109 strain. The obtained transformant was transferred to Hybond-N+ (Amersham Pharmacia Biotech), denatured, neutralized and then hybridized with the probe prepared in Example 5, (1) by using DIG-High Prime DNA Labeling & Detection Starter Kit I. At this time, a transformant that hybridized strongly and a transformant that hybridized weakly were recognized. Plasmid DNAs were prepared from these transformants and nucleotide sequences of inserts were determined. As a result, clones containing a gene showing high homology with respect to a known glutamine synthetase of coryneform bacteria could be obtained. The total nucleotide sequence of the insert of the latter was shown in Sequence Listing as SEQ ID NO: 1.

[0104] Open reading frames were deduced, and amino acid sequences deduced from the nucleotide sequences were shown in Sequence Listing as SEQ ID NOS: 2 and 3. Each of these amino acid sequences was compared with known sequences for homology. The used database was Genbank. As a result, it became clear that the amino acid sequences encoded by the both of the open reading frames were novel proteins of coryneform bacteria.

[0105] The nucleotide sequences and the amino acid sequences were analyzed by using Genetyx-Mac computer program (Software Development, Tokyo). The homology analysis was performed according to the method of Lipman and Pearson (*Science*, *227*, 1435-1441, 1985).

[0106] The amino acid sequence shown in Sequence Listing as SEQ ID NO: 2 showed 34.6%, 65.6% and 60% of homology with respect to already reported GS of *Corynebacterium glutamicum* (*FEMS Microbiology Letters*, 81-88, *(154)* 1997), GS of *Mycobacterium tuberculosis* (GenBank accession Z70692) and GS of *Streptomyces coelicolor* (GenBank accession AL136500), respectively (Table 6), and it was found to be an isozyme of GS of coryneform bacteria.

[0107] On the other hand, the sequence shown in Sequence Listing as SEQ ID NO: 3 showed 51.9% and 33.4% of homology with respect to the already reported ATase of *Mycobacterium tuberculosis* (GenBank accession Z70692) and ATase of *Streptomyces coelicolor* (GenBank accession Y17736), respectively (Table 7), and it was found to be ATase of coryneform bacteria. Therefore, it was found that, in the nucleotide sequence shown as SEQ ID NO: 1, the open reading frame coding for the amino acid sequence shown as SEQ ID NO: 2 was *glnA2*, and the open reading frame coding for the amino acid sequence shown as SEQ ID NO: 3 was *glnE*.

Table 6

Strain	Gene name	Amino acid Number	Homology
Brevibacterium lactofermen tum	*glnA2*	446 A.A.	--
Corynebacterium glutamicum	*glnA*	478 A.A.	34.6%
Mycobacterium tuberculosis	*glnA2*	446 A.A.	65.6%
Streptomyces coelicolor	*glnA*	453 A.A.	60.0%

Table 7

Strain Strain	Gene name	Amino acid number	Homology Homology
Brevibacterium lactofermentum	*glnE*	1045 A.A.	--
Mycobacterium tuberculosis	*glnE*	994 A.A.	51.9%
Streptomyces coelicolor	*glnE*	784 A.A.	33.4%

Example 6: Production of L-glutamine by ATase-deficient strain

[0108] Since the gene *glnE* coding for ATase was elucidated in the aforementioned Example 5, a *glnE*-deficient strain was constructed from the L-glutamine producing bacterium AJ12418. The specific procedure will be shown below.

[0109] First, PCR was performed by using chromosome DNA of *Brevibacterium flavum* ATCC 14067 strain as a template and the synthetic DNAs of SEQ ID NOS: 23 and 24 as primers to obtain a partial fragment of *glnE* gene. The produced PCR product was purified in a conventional manner, then blunt-ended and inserted into the HincII site of pHSG299 (Takara Shuzo). This plasmid was designated as pGLNE. Then, in order to delete a partial region of the *glnE* gene in this plasmid, pGLNE was digested with *Hinc*II and self-ligated, and the obtained plasmid was designated as pΔGLNE. This plasmid contained the 2341st to 4650th nucleotides of the nucleotide sequence shown in Sequence Listing as SEQ ID NO: 1, but it had deletion of about 300 bp from the 3343rd HincII recognition site to the 3659th HincII recognition site.

[0110] Since the above pΔGLNE does not contain a region that enables its autonomous replication within cells of coryneform bacteria, when a coryneform bacterium is transformed with this plasmid, a strain in which the plasmid is integrated into chromosome by homologous recombination may be produced as a transformant although it occurs at an extremely low frequency.

[0111] The L-glutamine producing bacterium, *Brevibacterium flavum* AJ12418, was transformed with the plasmid pΔGLNE at a high concentration by the electric pulse method, and transformants were obtained by using kanamycin resistance as a marker. Then, these transformants were subcultured to obtain strains that became kanamycin sensitive. Further, chromosomal DNAs of the obtained kanamycin sensitive strains were extracted, and PCR was performed by using each chromosomal DNA as a template and the synthetic DNAs shown in Secruence Listing as SEO ID NOS: 23 and 24 as primers to obtain partial fragments of the *glnE* gene. A strain of which PCR product did not provide about 300 bp fragment when it was digested with *Hinc*II was determined as a *glnE*-disrupted strain. This strain was designated as QA-T. Culture for L-glutamine production was performed in the same manner as described in Example 1,

(3) by using AJ12418 and QA-T strains. The results are shown in Table 8.

[0112] The QA-T strain showed improvement of L-glutamine accumulation compared with the AJ12418 strain. The results of measurement of the GS activity of these strains are also shown in Table 8. It was confirmed that the GS activity was improved in the QA-T strain compared with the AJ12418 strain.

Table 8

Strain	L-Gln (g/L)	GS activity (U/mg)	Culture time (hr)
AJ12418	39.0	0.03	70
QA-T	45.1	0.05	75

(EXPLANATION OF SEQUENCE LISTING)

[0113]

SEQ ID NO: 1: *glnA2* And *glnE* nucleotide sequences
SEQ ID NO: 2: *glnA2* amino acid sequence
SEQ ID NO: 3: *glnE* amino acid sequence
SEQ ID NO: 4: Primer N for *glnA* amplification
SEQ ID NO: 5: Primer C for *glnA* amplification
SEQ ID NO: 6: *glnA* 1st PCR primer NN
SEQ ID NO: 7: *glnA* 1st PCR primer NC
SEQ ID NO: 8: *glnA* 1st PCR primer CN
SEQ ID NO: 9: *glnA* 1st PCR primer CC
SEQ ID NO: 10: *glnA* 2nd PCR primer N
SEQ ID NO: 11: *glnA* 2nd PCR primer C
SEQ ID NO: 12: Primer N for *gdh* amplification
SEQ ID NO: 13: Primer C for *gdh* amplification
SEQ ID NO: 14: Primer N2 for *gdh* amplification
SEQ ID NO: 15: Primer C2 for *gdh* amplification
SEQ ID NO: 16: Primer M1 for *gdh* promoter mutation
SEQ ID NO: 17: Primer M4 for *gdh* promoter mutation
SEQ ID NO: 18: Primer N for *glnA* probe preparation
SEQ ID NO: 19: Primer C for *glnA* probe preparation
SEQ ID NO: 20: Wild-type *gdh* promoter sequence
SEQ ID NO: 21: Mutant type *gdh* promoter sequence

SEQ ID NO: 22: Mutant type *gdh* promoter sequence
SEQ ID NO: 23: Primer N for *glnE* disruption
SEQ ID NO: 24: Primer C for *glnE* disruption

SEQUENCE LISTING

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Leu Ser Leu Val Trp Asp Ala Val Leu Asp Ala Ala Leu Asp Ala Glu
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atc cgt gct gca ctt aac gat cca cag aaa cca gat cag cct ctg gcc  4321
Ile Arg Ala Ala Leu Asn Asp Pro Gln Lys Pro Asp Gln Pro Leu Ala
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aat att tct gtg atc ggc atg ggc cgt ttg ggt gga gca gaa ctt gga  4369
Asn Ile Ser Val Ile Gly Met Gly Arg Leu Gly Gly Ala Glu Leu Gly
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tac ggt tct gat gcc gat gtg atg ttt gta tgc gag ccg gta gcc ggt  4417
Tyr Gly Ser Asp Ala Asp Val Met Phe Val Cys Glu Pro Val Ala Gly
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Val Glu Glu His Glu Ala Val Thr Trp Ser Ile Ala Ile Cys Asp Ser
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Val Asp Ser Tyr Val Lys Tyr Tyr Glu Lys Trp Gly Glu Thr Trp Glu
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Ala Asp Thr Ile Ala Arg Pro Asp Pro Ser Thr Phe Gln Val Leu Pro
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Val Thr Met Pro Asp Gly Gln Pro Ser Phe Ser Asp Pro Arg Gln Val
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Lys Gly Gly Asp Pro Val Pro Phe Ser Thr Val Thr Met Gln Leu Ser
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995 1000 1005
Pro Asn Glu Tyr Gln Glu Tyr Leu Glu Asn Tyr Leu Lys Val Thr Arg
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Claims

1. A coryneform bacterium which has L-glutamine producing ability and has been modified so that its intracellular glutamine synthetase activity should be enhanced.

2. The bacterium according to Claim 1, wherein the glutamine synthetase activity is enhanced by increasing expression amount of a glutamine synthetase gene.

3. The bacterium according to Claim 2, wherein the expression amount of the glutamine synthetase gene is increased by increasing copy number of a gene coding for glutamine synthetase or modifying an expression control sequence of the gene so that expression of the gene coding for the intracellular glutamine synthetase of the bacterium should be enhanced.

4. The bacterium according to Claim 1, wherein the glutamine synthetase activity is enhanced by deficiency in activity control of intracellular glutamine synthetase by adenylylation.

5. The bacterium according to Claim 4, wherein the activity control of intracellular glutamine synthetase by adenylylation is defected by one or more of harboring glutamine synthetase of which activity control by adenylylation is defected, decrease of glutaimine synthetase adenylyl transferase activities in the bacterial cell and decrease of PII protein activity in the bacterial cell.

6. The bacterium according to any one of Claims 1-5, wherein the bacterium has been further modified so that its intracellular glutamate dehydrogenase activity should be enhanced.

7. The bacterium according to Claim 6, wherein the glutamate dehydrogenase activity is enhanced by increasing expression amount of a glutamate dehydrogenase gene.

8. The bacterium according to Claim 7, wherein the expression amount of the glutamate dehydrogenase gene is increased by increasing copy number of the gene coding for glutamate dehydrogenase or modifying an expression control sequence of the gene so that expression of the gene coding for the intracellular glutamate dehydrogenase of the bacterium should be increased.

9. A method for producing L-glutamine, which comprises culturing a bacterium according to any one of Claims 1-8 in a medium to produce and accumulate L-glutamine in the medium and collecting the L-glutamine.

10. A DNA coding for a protein defined in the following (A) or (B):

    (A) a protein that has the amino acid sequence of SEQ ID NO: 2,
    (B) a protein that has the amino acid sequence of SEQ ID NO: 2 including substitution, deletion, insertion, addition or inversion of one or several amino acid residues and has glutamine synthetase activity.

11. The DNA according to Claim 10, which is a DNA defined in the following (a) or (b):

    (a) a DNA containing the nucleotide sequence of the nucleotide numbers 659-1996 in the nucleotide sequence of SEQ ID NO: 1,
    (b) a DNA that is hybridizable with the nucleotide sequence of the nucleotide numbers 659-1996 in the nucleotide sequence of SEQ ID NO: 1 or a probe that can be prepared from the sequence under the stringent conditions and codes for a protein having glutamine synthetase activity.

12. A DNA coding for a protein defined in the following (C) or (D):

    (C) a protein that has the amino acid sequence of SEQ ID NO: 3,
    (D) a protein that has the amino acid sequence of SEQ ID NO: 3 including substitution, deletion, insertion, addition or inversion of one or several amino acid residues and has glutamine synthetase adenylyl transferase activities.

13. The DNA according to Claim 12, which is a DNA defined in the following (c) or (d):

(c) a DNA containing the nucleotide sequence of nucleotide numbers 2006-5200 in the nucleotide sequence of SEQ ID NO: 1,

(d) a DNA that is hybridizable with the nucleotide sequence of the nucleotide numbers 2006-5200 in the nucleotide sequence of SEQ ID NO: 1 or a probe that can be prepared from the sequence under the stringent conditions and codes for a protein having glutamine synthetase adenylyl transferase activities.

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European Patent Office
Office européen des brevets

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(71) Applicant: **Ajinomoto Co., Inc.**
Tokyo 104 (JP)

(72) Inventors:
- **Nakamura, Jun, c/o Ajinomoto Co., Inc.**
Kawasaki-shi, Kanagawa (JP)
- **Izui, Hiroshi, c/o Ajinomoto Co., Inc.**
Kawasaki-shi, Kanagawa (JP)
- **Moriguchi, Kayo, c/o Ajinomoto Co., Inc.**
Kawasaki-shi, Kanagawa (JP)
- **Kawashima, Hiroki, Ajinomoto Co., Inc.**
Kawasaki-shi, Kanagawa (JP)
- **Nakamatsu, Tsuyoshi,**
c/o Tokyo Denki University
Tokyo (JP)
- **Kurahashi, Osamu, c/o Ajinomoto Co., Inc.**
Kawasaki-shi, Kanagawa (JP)

(74) Representative: **HOFFMANN - EITLE**
Patent- und Rechtsanwälte
Arabellastrasse 4
81925 München (DE)

(54) Method for producing L-glutamine by fermentation and L-glutamine producing bacterium

(57) L-Glutamine is produced by culturing a coryneform bacterium which has L-glutamine producing ability and has been modified so that its intracellular glutamine synthetase activity should be enhanced, preferably which has been further modified so that its intracellular glutamate dehydrogenase activity should be enhanced, in a medium to produce and accumulate L-glutamine in the medium and collecting the L-glutamine.

EP 1 229 121 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 02 00 1993

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	DATABASE EMBL [Online] 24 January 2001 (2001-01-24) "sequence 95 from patent WO0100843" retrieved from EBI Database accession no. AX063813 XP002226627 -& WO 01 00843 A (BASF AG) 4 January 2001 (2001-01-04) ---	10,11	C12N15/52 C12N9/00 C12P13/14 C12N1/21 //C12R1/15
X	DATABASE EMBL [Online] 24 January 2001 (2001-01-24) "sequence 99 from patent WO0100843" retrieved from EBI Database accession no. AX063817 XP002226628 -& WO 01 00843 A (BASF AG) 4 January 2001 (2001-01-04) ---	12,13	
X	US 3 886 039 A (YOSHINAGA FUMIHIRO ET AL) 27 May 1975 (1975-05-27) * abstract * * column 1, line 65 - line 66 * * column 2, line 1 - line 4 * * column 4; example 1 * * column 5; table 4 * ---	1-9	TECHNICAL FIELDS SEARCHED (Int.Cl.7) C12N C12P
D,X	BOERMANN ET AL: "Molecular analysis of the Corynebacterium glutamicum gdh gene encoding glutamate dehydrogenase" MOLECULAR MICROBIOLOGY, BLACKWELL SCIENTIFIC, OXFORD, GB, vol. 6, no. 3, February 1992 (1992-02), pages 317-326, XP000864663 ISSN: 0950-382X * summary, first sentence * * page 317, right-hand column, reactions 1 and 2a * --- -/--	3,8	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	10 January 2003	Grötzinger, T

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
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Application Number
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Place of search	Date of completion of the search	Examiner
MUNICH	10 January 2003	Grötzinger, T

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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